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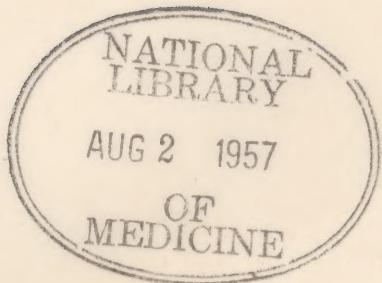
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Army Medical College Epidemiological
Research Report

Section 2 Number 135

Antibodies in Blood Eight Months After Inoculation
with Supersonic Wave-Treated Cholera Vaccine

Army Medical College Epidemiology Laboratory

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General

The effectiveness of the cholera vaccine in reducing the infection rate and mortality rate of the disease already has been demonstrated by FERRAN and later by KOLLE, SLATOGOROFF, STULIEN, ZAHALATUGS and LEINERIWADN. In our country this fact has been established through results of investigations performed in HYOGO Prefecture by MURATA and in OSAKA by HONURA during cholera epidemics.

The actual effectiveness of cholera-vaccine inoculations, however, has been evaluated in several different ways. KAMP and KERSTEN have claimed the effective period to be from three to four months, whereas KREIDEL, ROMER and HOFFMANN have claimed six months. The immunity titer disappears in seven to nine months, according to BALIKIN, and in one year, according to PAPAWAIKU and KUNRADI. HEITSCH has warned against exceeding the effective period and has recommended that inoculations be repeated every six months; the Germans and Austrians encountered success with this method during the World War.

Methods of manufacturing cholera vaccine are numerous, but in recent days the effectiveness of the supersonic wave-treated cholera vaccine has suddenly come into prominence. It has been reported already that in the immunization of humans the production of antibodies in the blood two weeks following the second inoculation proved superior to the controls. Since the continued effects of immune substances over an extended period among humans immunized with the supersonic wave-treated cholera vaccine remain unknown, tests to cover this phase have been ordered.

Chapter I. Test materials and procedure.

A. Test materials: The test sera consisted of sera taken on 9 Dec 39 from 34 out of 40 persons belonging to the Army Medical College Epidemiological Laboratory who, on 31 Mar 39 were inoculated with supersonic wave-treated cholera vaccines (hereafter referred to as U.S.V.) and from 22 out of 40 persons inoculated with control cholera vaccines (hereafter referred to as K.V.) prepared by the college.

Details on the above inoculation materials already have been described. (Ishii Force Bulletin II-80.)

Also included in the tests were sera taken on the same day from 152 persons (hereafter referred to as A.M.) not directly concerned with this experiment. The findings were not uniform as some of these persons had received cholera vaccine inoculations before March 1939 while others had no previous inoculations. Their sera were tested, however, to ascertain the general nature of antibodies in the blood.

The examinees were confined to those who from March 1939 to the day their blood was taken had not been inoculated with cholera vaccine. The sera were obtained after 5 cc of blood taken under sterile conditions from the median vein of each patient (empty stomach) was slant-coagulated in a sterile test tube. This was allowed to stand for 24 hours in a refrigerator (4°C) after which the serum underwent natural separation (some portions were removed by centrifugalization). Carbolic acid (0.5 per cent) was added and the sera were stored in a refrigerator.

B. Test procedure.

1. Agglutination reaction (Widal's reaction): The dilution of the test sera with a sterile physiological saline solution was successively doubled, starting with a 5-time dilution for the first test tube and ending with a 2,560-time dilution. The bacterial solution consisted of the Kitani strain cultured for 20 hours at 37°C in an agar medium and suspended at a ratio of 0.3 mg per cc in a physiological saline solution.

Following this, 1.0 cc of the bacterial suspension was added to 1.0 cc of the respective serum-dilutions mentioned above. This was shaken and incubated for two hours at 37°C. The results were observed on the following morning after it had been standing overnight at room temperature.

2. Complement fixation reaction: a. Preliminary test.

- (1) Hemolytic series: A goat hemolytic series was used. Four units of hemolysin-serum possessing a hemolytic titer of 6,400 times were employed.
(See Table 1.)

Table 1. Hemolysin value measurement test

Test tube number and hemolysin dilution	Hemolysin value							Control			
	I	II	III	IV	V	VI	VII	VIII	I	II	III
Hemolysin-serum	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Complement diluted 10 times	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
0.5 % sensitized blood cell solution	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Physiological saline solution	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.0	1.5	1.5
					37°C 2 hours						
Results	I	II	III	IV	V	VI	VII	VIII	IX	X	XI

- (2) Complement: Sera taken from more than 10 marmots were mixed (separated in refrigerator) and allowed to stand for more than three hours at room temperature. The complement titer was measured before each experiment. Dilution was approximately 10 times. (See Table 2.)

Table 2. Composite mean measure scores

Complement	8 X	10 X	12 X	14 X
	0.15	0.20	0.25	0.15
Physiological saline solution	1.35	1.20	1.25	1.25
			37° 1 hour	
5% sensitized blood cell solution	0.5	0.5	0.5	0.5
			37° 2 hours	
Results	X	L	X	X

(3) Antigen: The supersonic wave-treated antigen was obtained by culturing cholera bacteria (Kitami strain) in an agar medium for 18 hours at 37°C, suspending the bacteria in a physiological saline solution at a ratio of 10 mg per cc, subjecting 50 cc of the suspension to the action of 560 kc supersonic waves for 20 minutes in order to destroy the cells, and adding 0.5 per cent of carbolic acid. Complete hemolysis was displayed with a dilution of 8 times or more when the "self-retarding action" of this antigen was measured. The practical titer was set at 16 times. (See Tables 3 and 4.)

Table 3. Test on fixation strength of antigen and immune serum

Test tube number and antigen dilution	I	II	III	IV	V	VI	VII	VIII	Control		
									I	II	III
Antigen	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5			
Complement diluted 10 times	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Immune serum diluted 10 times	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5			
Physiological saline solution									1.5	1.0	0.5
5% sensitized blood cell solution	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Results									I	II	III

Table 4. Immunity & loss of natural antigen volume

Test tube number	X	11	111	1111	Y	111	1111	Centrifuged
antigen dilution	0	4	8	16	32	64	128	
antigen	0.5	0.5	0.5	0.5	0.5	0.5	0.5	
Creamed dilution 10 times	0.5	0.5	0.5	0.5	0.5	0.5	0.5	
Physiological saline solution	0.5	0.5	0.5	0.5	0.5	0.5	0.5	
								37°C 3 hour incubation
5% sensitized blood suspension	1.0	1.0	1.0	1.0	1.0	1.0	1.0	
								37°C 3 hour incubation
Results								

(4) Test sera: The test sera were rendered inactive by immersion in a 56°C water bath for 30 minutes.

(a) Main test: The dilution of the test sera with a physiological saline solution was successively doubled, starting with a 2.5-time dilution for the first test tube and ending with a 2,500-time dilution. Test serum volume was 0.5 cc of each dilution to which was added 0.5 cc of the complement and 0.5 cc of the antigen (starting with the second test tube). After incubating for one hour at 37°C 1.0 cc of a 5 per cent sensitized blood cell solution was added to each of the test tubes which were placed in the incubator for a two-hour period. These were allowed to stand at room temperature. The results were evaluated on the following morning.

(See Table 5.) The fixation strengths are indicated by symbols H (formerly ++), K (formerly + +), K (formerly + -), and L (formerly - -). (Symbol F c 1 has been added in this test.)

3. Test-tube bacteriolysis (Neisser-Wuchtery method).

The test sera were inactivated at a temperature of 56°C lasting for 30 minutes. The complement was obtained from more than 10 marmots. This was mixed and diluted 10 times before using. The bacterial suspension consisted of cholera bacteria (Kitani strain) cultured in an agar medium (PH 7.8) for 18 hours at 37°C and suspended in bouillon (PH 7.8). This was diluted to a point where the bacterial content equalled .000001 mg per cc and immediately used.

The dilution of the test sera with a physiological saline solution was successively doubled, starting with a dilution of 5 times and ending with a dilution of 2,500 times. Each test tube contained 1.0 cc of diluted sera to which 0.5 cc of the above bacterial suspension and 0.5 cc of the complement were added. The mixture was incubated for two hours (shaken during this period) at 37°C and later placed in a sterile Petri dish. Agar (approximately 50°C) was added in amounts of 15 cc each. Bacterial colonies were counted after culturing for 20 hours at 37°C. The bacteriolytic titer of the sera was taken as the highest serum dilution at which 10 or less cholera bacterium colonies were produced. Controls were also observed.

4. Immunization tests.

The cholera bacteria were of the Kitani strain which were cultured in an agar medium (PH 7.8) for 18 hours at 37°C and which displayed a minimum lethal dose of 0.2 mg against white German mice. Mice weighing from 10 to 13 g each were employed in the tests.

Intra-peritoneal injections were performed on these mice after 0.1 cc of fresh test sera and 0.4 cc of the cholera

bacterium suspension (0.3 mg of bacteria in 0.4 cc of physiological saline solution) were mixed in the hypodermic syringe. Observations were continued over a three-day period. Over 0.6 mg of cholera bacteria was required during the preliminary tests in producing total death.

Table 5. Main test procedure on complement fixation reaction.

Test tube number and serum dilution	I	II	III	IV	V	VI	Control		
	2.5	5	10	20	40	80	VII	VIII	IX
Serum volume (inactive)	0.5	0.5	0.5	0.5	0.5	0.5			.
Antigen diluted 16 times	-	0.5	0.5	0.5	0.5	0.5	-	-	0.5
Complement diluted 10 times	0.5	0.5	0.5	0.5	0.5	0.5	0	0.5	-
Physiological saline solution							1.5	1.0	1.0
	37°C 1-hour incubation								
St sensitized blood cell solution	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	37°C 2-hour incubation								
Results							H	L	L

Chapter II. Test results and summaries.

A. Agglutination reaction results and summary

(See Tables 6 and 7 below.)

Table 6. *Calibration reaction results*

Reaction condition		Reaction type						Total % error			
No.	Reaction condition	0	5	10	20	40	60	120	240	360	480
1.1.1.	Water positive	0	0	4	7	16	6	2	0	0	-
		0	0	0	0	0	0	0	0	0	22
1.1.2.	Water positive	1	-4	21	30	4	2	1	0	0	24
		2.02	12.12	33.32	30.3	12.12	6.66	2.02	0	0	24
1.1.3.	Water positive	16	43	49	23	15	3	1	0	0	152
		11.24	25.29	32.24	15.34	9.87	1.97	0.65	0	0	152

Table 7. Agglutination reaction results.

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Cases positive to the agglutination reaction at dilutions of 40 times and over totalled 57 per cent for the U.S.V., 32 per cent for the Z.V. and 12 per cent for the A.V. At dilutions of 10 times and over the positive cases totalled 100 per cent for the U.S.V., 84 per cent for the Z.V. and 69 per cent for the A.V. U.S.V. cases showing a negative agglutination reaction or positivity to the 5-time dilution were not encountered.

One case each was produced at the maximum agglutination value of 160-time dilution. The majority of the U.S.V. cases were positive to dilutions of 320 times and over, but in examining the drop in individual agglutination values after eight months had elapsed the values were found to be between 40 and 80 times. The values for Z.V. were still lower, indicating a drop to 10 to 20 times.

The U.S.V. indicated the highest average individual score, according to the agglutination reaction results, with a figure of 2.8, followed by Z.V. with 1.7 and by A.V. with 1.6. By score is meant the exponential score represented by the exponent of prime factor 2 for the absolute value of the antigen-antibody reaction titer possessed by the individual serum.

B. Complement fixation reaction results and summary:
Complement fixation strength was generally weak. In every instance the complement fixation titer was maximum at a 20-time dilution. Five cases (14.71 per cent) were indicated by U.S.V., one case (3.03 per cent) by K.V. and five cases (3.22 per cent) by A.N. (See Tables 8 and 9.)

Table 8. Complement fixation reaction results

Normal dilution	Yeast type						$\frac{1}{128}$ $\frac{1}{256}$
	0	5	10	20	40	-	
Marker positive	12	0	9	5	0	0	$\frac{1}{12}$
%	33.33	25.53	25.67	11.71	0	0	$\frac{1}{256}$
Marker positive	13	14	5	1	0	0	$\frac{1}{25}$
%	39.39	42.42	15.38	3.45	0	0	$\frac{1}{256}$
Marker positive	74	45	20	5	0	0	$\frac{1}{256}$
%	42.02	22.95	19.05	3.23	0	0	$\frac{1}{256}$

Table 9. Complement fixation reaction results.

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Definite results showing a decrease in individual complement fixation strengths were not observed. The average individual score was 0.56 point for U.S.V., 0.21 point for X.V. and 0.26 point for A.N., the latter two being less than one half of U.S.V.

C. Test-tube bacteriolysis results and summary: (See Tables 10 and 11.)

Table 10. Selected results from 1966

Serum type		1966										Total score total cases	
		5	9	30	20	40	60	100	320	640	1280	2560	5120
E. V.	Positive cases	2	1	2	1	2	2	6	5	5	4	4	172
E. V.	Positivity %	5.00	6.67	20.00	10.00	5.00	5.00	17.50	16.71	11.71	11.71	11.71	172
A.U.	Positive cases	2	2	2	2	2	2	6	6	6	6	6	143
A.U.	Positivity %	6.06	15.38	6.06	6.06	6.06	6.06	15.38	15.38	15.38	15.38	15.38	143
A.U.	Positive cases	22	16	9	5	17	20	11	11	19	16	7	52
A.U.	Positivity %	14.47	15.51	5.92	3.29	11.11	13.33	11.11	6.50	11.50	10.53	6.50	52

Table 11. Bacteriolysis test results



More than half (53 per cent) of the U.S.V. cases indicated a bacteriolytic titer of 320 times and over. Approximately half (48 per cent) of the E.V. cases indicated a titer of 80 times and over, not differing to any great extent from that displayed by A.N.

Fluctuations in individual bacteriolytic titer after eight months were observed, but because of the insufficient amount of sera and the small number of examined cases in the initial series a standard for comparison could not be established. It was observed, however, that eight months after inoculation the U.S.V. retained a distinctly higher amount of hemolysin as compared to the control.

The highest average individual score was displayed by U.S.V. with 5.3 points, followed by E.V. with 3.5 points and A.N. with 3.2 points.

D. Immunization test results and summary: The immunization tests were performed on laboratory animals corresponding in number to the 32 persons, 29 persons and 141 persons from whom the U.S.V., E.V. and A.N. test sera, respectively, were derived. (See Tables 12 and 13.)

Table 32. Immunization test results

Days observed		1	2	3	Total
U.S.A. (From 34 cases)	Deaths	8	6	1	15
	Death rate (%)	25	16.75	3.13	46.85
C.V. (From 53 cases)	Deaths	14	5	1	20
	Death rate (%)	42.27	17.24	3.05	62.97
A.M. (From 152 cases)	Deaths	83	21	10	122
	Death rate (%)	52.27	13.90	12.77	66.52

Table 13. Immunization test results

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The death rate of animals employed in the tests and inoculated with K.V. and A.M. practically reached or exceeded one half their number on the first day, whereas the rate for U.S.V. was only 26 per cent. The total covering the three-day period reveals a 47-per cent rate for U.S.V., a 69-per cent rate for K.V. and an 87-per cent rate for A.M.

Deaths among the infected animals were the highest on the first day, but decreased on the second and third days, in that order.

These results show the U.S.V. serum to possess the highest immunization strength followed by the K.V. serum; the A.M. serum displayed extremely weak immunization strength.

Chapter III. Conclusion

A. Comparative studies were made on the antibodies contained in human blood eight months after inoculations of supersonic wave-treated cholera vaccines and control cholera vaccines (manufactured by Army Medical College) were made on the same day. In addition the antibodies contained in the blood of persons not connected directly with the above experiment were studied.

B. The sera from persons who had been inoculated with supersonic wave-treated vaccines were found to retain the original agglutinin, complement fixation substances, bacteriolysin and immune substances eight months after the inoculation. The amount retained greatly surpassed that retained in persons who had been inoculated with the control cholera vaccine (manufactured by Army Medical School).

C. The antibodies contained in the blood of persons who had been inoculated eight months previously with the control cholera vaccine (manufactured by Army Medical School) gave the impression of being slightly superior in number to those of ordinary persons. The results, however, indicated no great difference between the two.

Supplement

An opportunity to examine the blood types of a large number of persons was provided. The results of this examination have been added to the main text as a supplement.

The standard serum prepared by the Army Medical College Epidemiological Section was dissolved in a physiological saline solution according to an established rule. Blood cells of the highest possible freshness were allowed to act upon this. Blood cell agglutination was examined and the results were determined within a three-minute period. (See Tables 14, 15 and 16 and Supplementary Table 1.)

Supplementary Table 1. Blood type



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Key

- (1) Blood type

A comparative study of the individual antibody productivity performed concurrently with inoculations (based on blood types) of supersonic wave-treated cholera vaccine and control cholera vaccine revealed no particularly marked differences. (See supplementary Table 2 for comparative results.)

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Blood type	Serum number	U.S.Y.						K.V.							
		Agglutination reaction		Complement fixation reaction		Bacteriolysis		Immunity	Agglutination reaction		Complement fixation reaction		Bacteriolysis		
		8 mos. previous	Present	8 mos. previous	Present	8 mos. previous	Present		8 mos. previous	Present	8 mos. previous	Present	Immunity		
O	Results	28	320 - 40	10 - 20	-	20	(+)	36	640 - 0	40 -	5	1280 - 40	40	(-)	
		44	40 - 40	5 - 20	-	1280	(+)	57	640 - 10	20 -	10	1280 - 10	10	X	
		165	640 - 40	160 - 20	0 -	640	(+)	75	640 - 20	20 -	0	-	5	(-)	
		167	640 - 20	20 - 0	-	60	(-)	96	160 - 5	5 -	0	-	45	(-)	
		178	320 - 20	40 - 5	-	640	(+)	106	320 - 20	20 -	0	-	20	(-)	
		179	- - 20	- - 5	-	0	(-)	189	- - 40	- -	5	- -	5	X	
		182	640 - 40	40 - 5	640	320	(+)	146	80 - 20	5 -	5	-	80	(+)	
		191	640 - 40	40 - 0	0 -	160	(+)	149	- - 10	- -	20	-	5	(-)	
		6	- - 40	- - 5	-	2560	(-)	187	640 - 10	20 -	5	640 - 640	160	(-)	
		22	- - 40	- - 10	-	1280	(+)	73	640 - 10	40 -	10	640 - 640	40	(-)	
		65	- - 10	- - 5	-	0	(-)	55	- - 80	- -	0	-	40	(+)	
		80	- - 80	- - 0	-	160	(+)								
		126	- - 40	- - 5	-	160	(+)								
		180	640 - 10	- - 10	0 -	2560	(+)								
		134	- - 40	- - 10	-	320	(+)								
Individual average	Titer score points	485 - 34	49 - 6	160	678	11		Titer score points	470 - 20	20 -	5	1024 - 40	40	2	
		6.25 - 2.53	2.63 - 0.47	175	540	11			6.25 - 1.55	1.63 -	0.36	7.60 - 2.18	2.18	9	
A	Results	16	640 - 40	20 - 0	-	160	(+)	27	320 - 20	10 -	5	- -	640	(-)	
		18	640 - 20	20 - 5	-	2560	(-)	74	164 - 40	10 -	5	- -	160	(+)	
		28	320 - 20	20 - 0	-	320	(-)	124	640 - 5	20 -	5	640 -	1280	(+)	
		99	320 - 0	40 - 5	-	10	(X)	133	160 - 20	5 -	0	- -	1280	(+)	
		127	320 - 20	10 - 20	-	320	(+)	217	160 - 20	5 -	5	- -	5	(-)	
		132	320 - 40	20 - 0	-	160	(+)	197	320 - 5	20 -	5	- -	0	(-)	
		135	320 - 40	20 - 20	-	640	(-)	175	80 - 20	0 -	0	- -	160	(-)	
		4	- - 20	- - 10	-	40	(-)	188	160 - 10	5 -	0	- -	80	(-)	
		70	- - 20	- - 10	-	320	(+)	35	- - 10	- -	0	- -	5	(-)	
		125	- - 20	- - 10	-	80	(-)								
		131	- - 80	- - 10	-	320	(+)								
		72	- - 80	- - 0	-	160	(+)								
	Individual average	Titer score points	411 - 40	21 - 7	0	479	5		Titer score points	250 - 17	9 -	4	640 - 401	401	7
			6.29 - 2.75	2.00 - 0.66	0	5.5	11			5.37 - 1.44	7.50 -	0.11	7	4.11	7
B	Results	47	160 - 160	10 - 10	-	1280	(+)	21	640 - 20	20 -	10	1280 -	160	(+)	
		128	640 - 10	80 - 0	0 -	5	(-)	33	320 - 10	10 -	0	-	40	(-)	
		150	160 - 80	10 - 10	-	640	(-)	58	80 - 5	5 -	10	- -	80	(+)	
		151	320 - 40	2 - 0	-	10	(-)	94	640 - 40	40 -	0	640 -	40	(-)	
		154	640 - 10	-	0	80	(X)	168	160 - 10	8 -	0	320 -	80	(-)	
		301	640 - 40	80 - 0	0	80		176	640 - 10	40 -	5	- -	640	(-)	
								25	- - 5	- -	0	- -	20	(-)	
								56	- - 5	- -	0	- -	10	(-)	
								71	- - 160	- -	0	- -	160	(-)	
								88	- - 20	- -	0	- -			
	Individual average	Titer score points	4.62 - 62	37 - 3	213	342	1		Titer score points	4.00 - 28	19 -	4	747 - 7	126	3
			6.17 - 3.17	2.33 - 0.33	2.33	4.60	7			1.43 - 1.63	1.5 -	0.27	7	3.54	10
AB	Individual average		320 - 80	10 - 20	-	2560	(-)	136	320 - 40	10 -	5	-	640	X	
			6 - 4	1 - 2	-	91	1	153	640 - 10	20 -	0	640	(+)		

Table 14. Results on persons inoculated with U.S.V.

Note: Persons inoculated with U.S.V. but who were absent from the first test have been listed in this table starting with Serum Number 1.

Table 15. Test results on persons inoculated with K.V.

Table 16-1. Test & results with A.M.

Table 16-2. Test results with A.H. (Cont'd)

Table 14-2. Test results with A.M. (Cont'd)

O	-	65
A	-	53
B	-	23
AB	-	11